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Production of electrospun gelatin nanofiber by water-based co-solvent approach

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Abstract In this study, gelatin, was successfully electrospun from a newly developed water-based co-solvent composed of ethyl acetate and acetic acid in water. Since natural polymers including gelatin exhibit limited solubility in water, toxic or highly acidic solvents are normally used to dissolve them for electrospinning. Instead of using those solvents, we used ethyl acetate in concert with acetic acid in water, and investigated the beneficial effect of its use in terms of the spinnability of the nanofiber and the acidity of the solvent. The replacement of acetic acid with ethyl acetate was observed to improve the spinnability of the nanofiber by reducing the surface tension of the solution as well as to increase the pH of the solvent significantly. The optimal composition of the co-solvent was found to correspond to a ratio of ethyl acetate to acetic acid of 2:3. Under this solvent condition, the gelatin could be dissolved at concentrations of up to ~11 wt% and electrospun successfully to produce nanofibers with various diameters (47–145 nm on average) depending on the gelatin concentration. The water-based co-solvent method proposed herein may be useful for generating other nanofibrous natural polymers as well as being applicable in delivery systems for bioactive molecules within the nanofiber matrices.

Introduction

The artificial replacement of damaged and diseased tissues constitutes a promising avenue of research in the field of regenerative medicine. Over the past decades, significant progress has been made in the development of many tissue-regenerative biomaterials, for example new bioactive compositions have been produced and novel processes and methodologies have been designed. In this context, the recent advent of nanotechnology has enriched the field of biomaterials and tissue engineering [1–3].

At the heart of these developments is the electrospinning process, which plays a crucial role in the generation of nanoscale fiber of biomaterials ranging from polymers and ceramics to their composites. In particular, many natural and synthetic polymers, such as proteins, polysaccharides and poly (α-hydroxyl) acids, have been transformed into fibers with diameters in the range of tens to hundreds of nanometers [3–6]. This type of nanofibrous structure is regarded as a promising architecture in the sense that the natural extracellular matrix (ECM) exhibits a fibrous structure with diameters on the nanoscale, which is far smaller than can be achieved with conventional processing methods [7, 8].

Among the various materials that can be used to produce electrospun fibers, natural polymers including proteins (gelatin, collagen and silk fibrinogen) and polysaccharides (chitosan, hyaluronic acid and cellulose) are difficult to electrospin, because of their high viscosity and low solubility in general organic solvents. There is a limited number of solvents, including 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoroacetic acid, that can be used to dissolve natural polymers [7–10]. However, these solvents are highly toxic, so that it is preferable to use nontoxic aqueous systems for potential biomedical applications.

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Unfortunately, aqueous systems are known to be unsuitable to electrospin natural polymers, because of the high surface tension or low volatility of the aqueous solution. In recent studies, a modified solvent using polyethylene oxide (PEO) was used in the electrospinning of chitosan or hyaluronic acid [11, 12]. Some studies avoided the use of PEO, by introducing acidic solvents, wherein acetic acid or formic acid was added to dissolve natural polymers such as chitosan and gelatin [13, 14]. However, these solvents generally resulted in some degradation of the polymers owing to their acidity [14]. Recent studies have pointed to the urgent necessity to develop nontoxic aqueous solvents which can be used for the electrospinning of natural polymers [14, 15].

In order to meet this challenge, we attempted to create natural polymer nanofibers (particularly the protein system described herein) by using a nontoxic and less acidic solvent. To this end, a co-solvent, namely ethyl acetate in concert with acetic acid in water was used in order to dissolve the polymer. In particular, this newly designed co-solvent is considered to possess the solution properties, which are needed to dissolve the proteins and allow them to be electrospun successfully. We examined the role of the composition of the co-solvent in the successfulness of the electrospinning process using gelatin as the polymer. Moreover, the optimal electrospinning conditions required to generate the nanofiber without the formation of beads were determined.

Materials and methods

Materials

Gelatin (type B, from bovine, Sigma), glacial acetic acid (99.99%, Sigma) and ethyl acetate (anhydrous, 99.8%, Sigma) were used as received, without any treatment or further purification.

Solvents and electrospinning

To electrospin the gelatin, various sets of solvents were composed using acetic acid, ethyl acetate and distilled water, as summarized in Table 1. Ten wt% of gelatin was dissolved in each acidic solvent with or without ethyl acetate. Solvents 1–5 consisted of various ratios of distilled water to acetic acid (water:acetic acid = 1:9 to 9:1) without any ethyl acetate. In solvents 6–13, the ethyl acetate was added to the acetic acid solvent at different ratios (acetic acid:ethyl acetate = 4:1 to 1:1) while keeping the ratio of water to acetic acid + ethyl acetate fixed (water/acetic acid + ethyl acetate = 1:2 for sets 6–9 and 1:3.5 for sets 10–13). As a reference, gelatin was dissolved in the typically used toxic solvent, hexafluoro-2-propanol (HFP) at concentrations of up to 0.1 g/ml. All the solutions were incubated at 37 °C for 12 h prior to electrospinning.

The prepared gelatin solution was loaded into a syringe (with a capacity of 5 ml and a needle diameter of 500 μm) and injected onto a metal collector under a high direct current field strength (12 kV/8 cm) at an injection rate of 0.06 ml/h. The electrospun samples were dried under vacuum at 37 °C overnight to evaporate the remnant solvent.

Characterization

The surface tension of the solution was analyzed by means of the pendant drop method using a contact angle and surface tension analyzer (Phoenix 300, SEO, Korea). The droplet image of each solution was observed using a viewing system until the equilibrium shape was achieved at 25 °C. The surface tension was calculated by the equation,

Table 1 Composition of gelatin solution used for electrospinning

Number	Gelatin (wt%)	Water (wt%)	Acetic acid (A.A.) (wt%)	Ethyl acetate (E.A.) (wt%)	Water: A.A + E.A.	A.A:E.A
1	10	81	9	0	9:1	–
2	10	63	27	0	7:3	–
3	10	45	45	0	1:1	–
4	10	27	63	0	3:7	–
5	10	9	81	0	1:9	–
6	10	30	60	0	1:2	–
7	10	30	48	12	1:2	4:1
8	10	30	36	24	1:2	3:2
9	10	30	30	30	1:2	1:1
10	10	20	70	0	1:3.5	–
11	10	20	56	14	1:3.5	4:1
12	10	20	42	28	1:3.5	3:2
13	10	20	35	35	1:3.5	1:1

The concentration of gelatin was fixed and the ratio of the three solvents was changed

$\gamma = g\rho d_e^2/H$, where g is the acceleration due to gravity, ρ is the difference in density of the air and solution, d_e is the equatorial diameter of the droplet and H is the shape factor, as referenced in [16]. The pH of the solvents was measured using a pH meter (SunTex Co., Taiwan) at room temperature.

The morphology of the electrospun nanofiber was examined using field-emission scanning electron microscopy (FE-SEM, JEOL, Japan). The diameter of the nanofiber was measured from 30 arbitrary selected samples and averaged. The thermal properties of the electrospun gelatin were analyzed using differential scanning calorimetric analysis (DSC Q-1000, TA Instrument, UK). The measurement was conducted with a scanning speed of 20 °C/min, with flowing nitrogen gas at a rate of 50 ml/min.

Results and discussion

Properties of co-solvent

Successful electrospinning requires that certain parameters be suitably controlled, such as the solute solubility in the solvent, the evaporation rate of the solvent, and the viscosity and surface tension of the solution. Amongst these parameters, the surface tension is considered to play a major role and, in this study, it is adjusted by the addition of ethyl acetate. It is generally considered that the high surface tension of a solution inhibits the electrospinning process, thereby resulting in the instability of the jets and the generation of sprayed droplets [17]. Although it is generally considered that the reason for the difficulty encountered in electrospinning water-containing solvents is the low evaporation rate of water, its extremely high surface tension (~74.2 mN/m) also contributes in large part to their poor spinnability [18–20]. In the case of natural polymers, including proteins, the pure water system cannot be used in electrospinning because of the limited dissolvability of proteins. On the other hand, acidic solvents, mainly acetic acid in the case of gelatin, constitute another possible choice to dissolve proteins. Moreover, acetic acid is beneficial in that it reduces the surface tension of the solvent (the surface tension of acetic acid is ~28.8 mN/m) [19, 20].

Figure 1 shows the variation in the surface tension of the gelatin solutions dissolved in the solvents composed of water, acetic acid and ethyl acetate at different concentration ratios. As illustrated in this figure, the acetic acid present in the water decreased the surface tension of the solution effectively. More intriguingly, the partial replacement of acetic acid by up to 50 wt% of ethyl acetate decreased the surface tension even more, demonstrating that ethyl acetate is more effective in decreasing the surface tension of water than acetic acid. As such, it is considered that the surface tension of ethyl acetate is lower than that of acetic acid (~28.8 mN/m), as well as being far less than that of water (~74.2 mN/m). The lowest surface tension that could be achieved in this study was ~20 mN/m, which was accomplished by replacing 50 wt% of acetic acid with ethyl acetate at a water content of 20 wt%. However, increasing the amount of ethyl acetate to over 50 wt% made the solution turn into a gel with time. Moreover, at the water content of less than 20 wt% it was difficult to mix the ethyl acetate with acetic acid in water homogeneously.

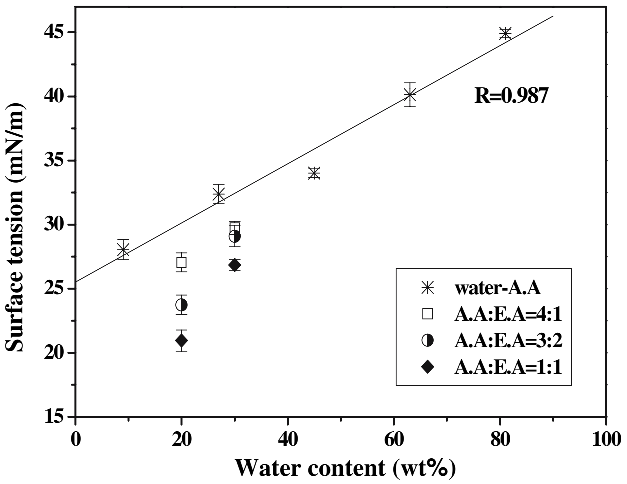

Fig. 1 Surface tension of the gelatin solutions dissolved in water-based co-solvents consisting of water, acetic acid and ethyl acetate at different ratios. As the amount of acetic acid increased (*symbol asterisk*), the surface tension of the solution decreased linearly. The replacement of acetic acid with up to 50 wt% of ethyl acetate (*other symbols*) decreased the surface tension even more

Spinnability of gelatin solution

The SEM morphologies of the gelatin electrospun from the water-based solvents containing various concentrations of acetic acid (without ethyl acetate) are shown in Fig. 2. When the water to acetic acid ratio was 7:3 (Fig. 2A), mostly beads were produced. However, when the acetic acid content was increased (a water to acetic acid ratio of 1:1 in Fig. 2B), fibrous products started to be generated. When the concentration of acetic acid was further increased (a water to acetic acid ratio of 3:7 in Fig. 2C), mainly nanofibers were produced. However, in this case, some beads were still formed. When the proportion of acetic acid was increased to 81% (a water to acetic acid ratio of 1:9 in Fig. 2D) nanofibrous gelatin was successfully generated without the formation of beads. The SEM morphologies in Fig. 2 showed a similar trend, as observed in the reduction of the surface tension shown in Fig. 1.

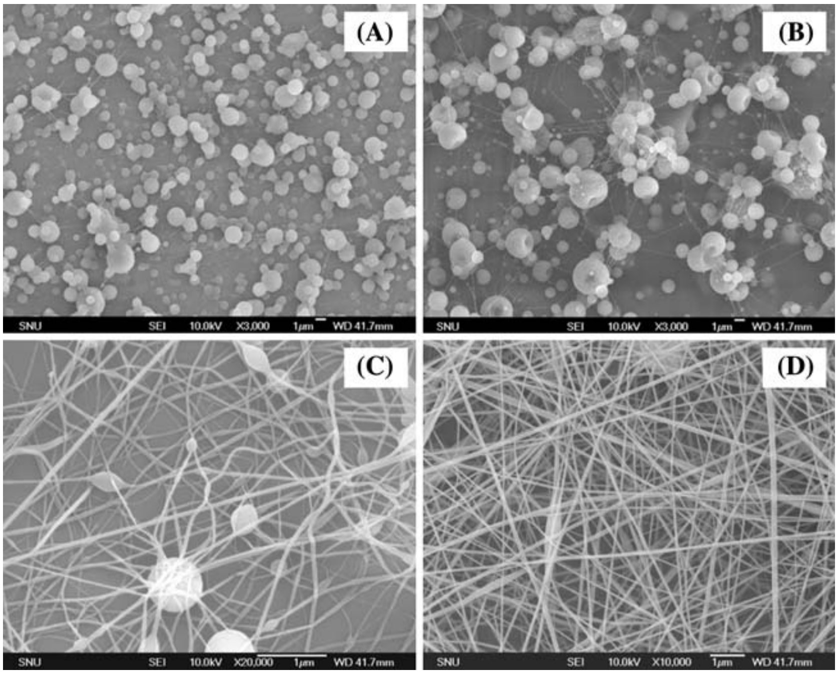

Fig. 2 SEM morphologies of gelatin solutions with (**A**) water:acetic acid = 7:3, (**B**) 1:1. (**C**) 3:7, and (**D**) 1:9. In the case of water:acetic acid = 9:1, the solution could not be electrospun. As the amount of acetic acid increased, the solution was able to be electrospun better

Figure 3 shows the variation in the SEM morphologies of the gelatin electrospun from the water-based co-solvents containing acetic acid as well as ethyl acetate. At a fixed water content (30 and 20 wt%), the effect of the ethyl acetate added in replacement of acetic acid is illustrated. At a water content of 30 wt% (Fig. 3A–D), it was observed that as the concentration of ethyl acetate increased the gelatin nanofiber was better able to be electrospun. Although some beads were generated from the solvent containing a high concentration of ethyl acetate, the nanofibrous morphology was well developed. At a lower water content (20 wt%, Fig. 3E–G, a similar trend was observed with respect to the effect of ethyl acetate. The generation of the nanofiber (without bead formation) appeared to be better at a water content of 20 wt% than at a water content of 30 wt% (the effect of the water content was illustrated in Fig. 2), where a lower concentration of ethyl acetate was required to create a well-developed nanofibrous gelatin morphology. Based on this study, the optimal solvent composition for the electrospinning of gelatin is considered to be a ratio of acetic acid:ethyl acetate of 3:2 in 20 wt% water (Fig. 3G). In this condition, the gelatin nanofibers were well-stretched without any beads being formed.

pH consideration of acidic solvent

Natural polymers including proteins dissolve to only a limited extent in pure water, and this is also the case for gelatin. In the case of polysaccharides such as chitosan and glucosaminoglycans, this solubility limitation in water was reported to be more severe. Acidic solvents such as acetic acid and boric acid, although effective in dissolving these polymers, should be used with caution, because their high acidity results in the degradation of the native characteristics of the polymers. Moreover, when incorporating bioactive molecules such as antibiotics, enzymes and genes within the polymeric matrices, such a harsh acidic condition would be detrimental to the biological function of the bioactive agents, consequently limiting their potential uses [21].

We measured the pH of the gelatin solutions used for the electrospinning process, as shown in Fig. 4. The addition of acetic acid to water decreased the pH dramatically. Only those solvent compositions with a water content of less than 20 wt% could be used for the electrospinning of gelatin. However, in this composition range the solution was highly acidic (the pH was less than ~0.2 and sometimes below 0). Although the spinnability was good, in this composition range concerns are raised with regards to the stability of gelatin and possible use of other bioactive molecules. However, the use of ethyl acetate as a co-solvent in replacement of a partial amount of acetic acid significantly increased the pH of the solution. Along with its other roles, as discussed in the previous sections, ethyl acetate also proved to be beneficial in increasing the pH of the acidic solution, thus facilitating the use of other bioactive molecules in concert with the nanofibrous protein matrices. Although the pH range observed herein was still acidic (the highest pH observed in this study was ~1.5) in the presence of ethyl acetate, its role in increasing the pH makes it very promising for future applications involving

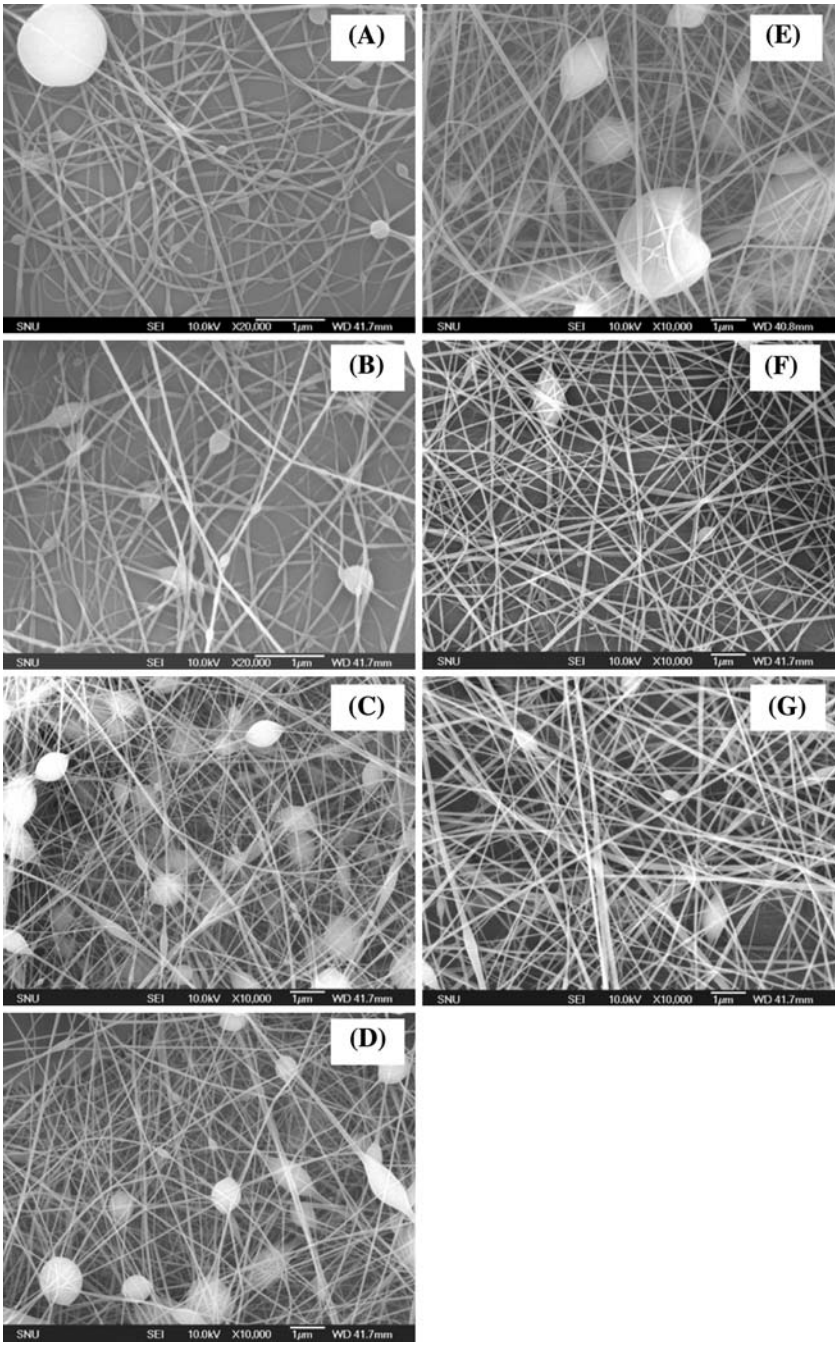

Fig. 3 SEM morphologies of gelatin solutions composed of water, acetic acid and ethyl acetate. The water content was fixed and the ratio of acetic acid to ethyl acetate was varied: 30 wt% water containing (**A**) only acetic acid, (**B**) acetic acid:ethyl acetate = 4:1, (**C**) 3:2. (**D**) 1:1, and 20 wt% water containing (**E**) only acetic acid, (**F**) acetic acid:ethyl acetate = 4:1, (**G**) 3:2

other nanofiber systems, such as other proteins, polysaccharides, glucosaminoglycans, etc., as well as in delivery systems containing bioactive molecules, and this remains as a future study.

Stability of electrospun gelatin

In order to investigate the stability and degradation of the gelatin nanofiber obtained from the acidic solvent, we performed a DSC analysis, as shown in Fig. 5. Three sets of gelatin nanofibers obtained from different solvents (HFP, acetic acid in water, and ethyl acetate added to acetic acid in water) are compared. It is well recognized that the raw gelatin powder shows the appearance of an endothermic peak at approximately 220 °C, which is known to correspond to the decomposition temperature of gelatin [14, 22]. Surprisingly, the data on the gelatin electrospun from HFP, which is the most widely used solvent to dissolve proteins including gelatin, exhibited no distinct thermal peak at this temperature (Fig. 5A). Rather, only the broad trace of an endothermic region appeared at ~200 °C. In the gelatin obtained from the 63 wt% acetic acid

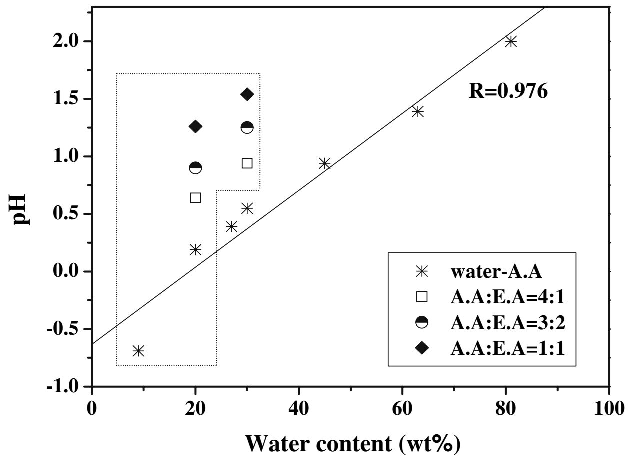

Fig. 4 pH of the gelatin solutions dissolved in water-based co-solvents consisting of water, acetic acid and ethyl acetate at different ratios. Although the addition of acetic acid was productive in the electrospinning of the water-based system, the increase in acetic acid decreased the pH markedly (linearly proportional). However, the replacement of acetic acid with ethyl acetate increased the pH significantly. Data inside the dashed box include the compositions for which electrospinning is possible

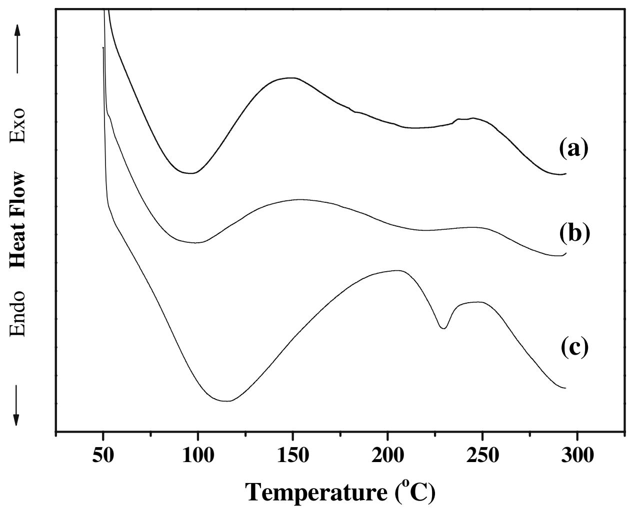

Fig. 5 DSC data on the gelatin nanofibers electrospun using different solvents: (A) HFP, (B) water:acetic acid = 3:7, and (C) water:acetic acid:ethyl acetate = 0.2:0.42:0.28

solvent, a similar trend was observed (Fig. 5B). This result corresponds well to the previous study by Ki et al., wherein gelatin was obtained from a formic acid solvent [14]. However, the gelatin nanofiber obtained from the ethyl acetate-added co-solvent (acetic acid:ethyl acetate = 3:2) showed a sharp decomposition peak at ~225 °C, which is characteristic of the initial gelatin powder (Fig. 5C). This DSC result supports the hypothesis that the solvents used to dissolve gelatin, particularly the acidic solvents used herein, have some detrimental effect on the structural properties of gelatin. This issue should also be carefully considered in other natural polymer systems which are difficult to dissolve for electrospinning.

Gelatin concentration effect

Using the newly composed solvent, gelatin nanofibers with various diameters could be produced by controlling the concentration of gelatin. Using the predetermined optimal solvent composition (ethyl acetate:acetic acid = 3:2 in 20 wt% water), the gelatin concentration was varied from 6 to 12 wt%.

The SEM morphologies of the electrospun gelatin fiber obtained at various concentrations are shown in Fig. 6. With a gelatin concentration of 6 wt%, mostly beads were formed, and this was attributed to the viscosity of the solution being too low to generate continuous fiber (Fig. 6A). As the concentration was increased to 7 and 8 wt%, the formation of beads was reduced and continuous nanofiber was generated (Fig. 6B–C). When the gelatin concentration was 9 wt%, the nanofibers were clearly generated (Fig. 6D). When the gelatin concentration was further increased to 10 and 11 wt%, the nanofibers were also generated well and their diameter increased markedly. At the highest concentration (12 wt%), it was difficult to completely dissolve the gelatin, as this concentration exceeds the solubility limit. Figure 7 shows the diameter of the nanofibers with respect to the gelatin concentration. As was observed in the SEM morphology, the diameter increased with increasing concentration of gelatin. The diameters obtained were in the range of several tens to hundreds of nanometers (from 47 to 145 nm on average). In this study, only the concentration of gelatin was varied, as this was observed to be the most dominant factor determining the diameter of the nanofiber.

It is anticipated that this method of electrospinning gelatin using a water-based co-solvent could be applied to other natural polymeric systems which are scarcely soluble in water solvent, including polysaccharides, glucosaminoglycans, other proteins, and their mixtures. Moreover, more extended applications of these polymeric matrices are to be expected, such as drug delivery systems, since the bioactive molecules to be incorporated normally favor less toxic and less acidic solvents.

Conclusion

A novel co-solvent made of ethyl acetate, acetic acid and water was used to electrospin nanofibers of gelatin. The addition of ethyl acetate improved the spinnability of the nanofiber and reduced the acidity of the solvent

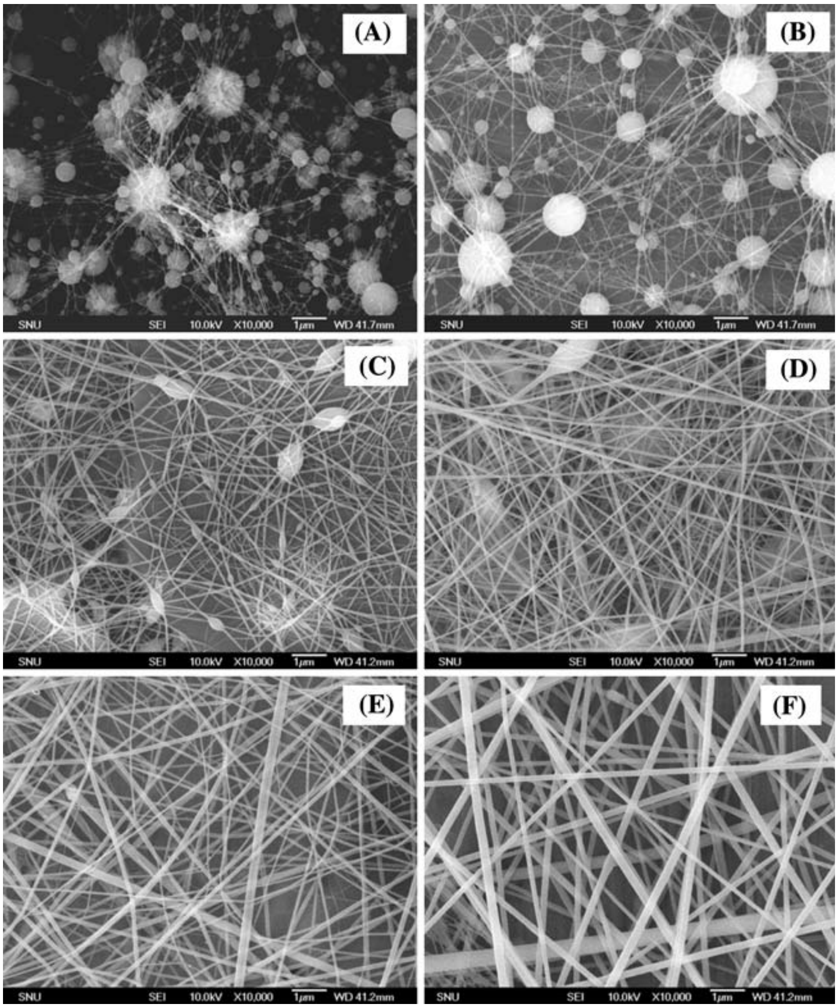

Fig. 6 SEM morphologies of the gelatin electrospun with different gelatin concentrations: (**A**) 6 wt%, (**B**) 7 wt%, (**C**) 8 wt%, (**D**) 9 wt%, (**E**) 10 wt% and (**F**) 11 wt% using a solvent of acetic acid:ethyl acetate = 3:2 in 20 wt% water

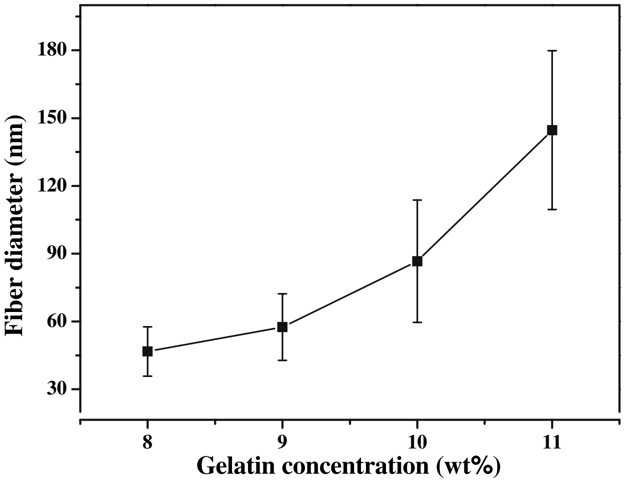

Fig. 7 Diameters of the gelatin nanofibers obtained by varying the gelatin concentration from 8 to 11 wt%. The solvent composition was acetic acid:ethyl acetate = 3:2 in 20 wt% water. The diameters were measured from the SEM images of 30 arbitrarily selected samples and then averaged

significantly. Within the co-solvent gelatin could be dissolved at concentrations of up to ~11 wt%, and the generated nanofibers had a range of diameters (47–145 nm on average). The co-solvent developed herein is considered to be highly useful for electrospinning other natural polymers which are limitedly soluble in water-based solvents.

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